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# The effect of imipramine hcl on haemolysis of horse erythrocytes

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**Abstract**

The mechanism of action of tricyclic antidepressant (TCAs) is not fully understood. The effect of the TCA imipramine on the haemolysis of erythrocytes was investigated, focussing on dosage and incubation time. The method used a drug free red blood cell (RBC) haemolysis assay (Watts & Handy, 2007) comparing experimental drug concentrations ranging from 0.0904 mg/ml – 90.4 mg/ml. Incubation time effects were also investigated, and haemolysis increased with decreasing osmolarity. Compared with the control the percentage haemolysis increased significantly with higher concentrations of imipramine, but was reduced by 0.0904 mg/ml imipramine. Haemolysis also increased with longer incubation time in both experimental and drug-free trials. Findings show that imipramine has a haemolytic effect on RBCs that is both incubation time and concentration dependant. Imipramine has a stabilising effect on RBCs at 0.0904 mg/ml around critical micelle concentration which could involve surface-drug-action and can be explained by Kesting's liquid membrane hypothesis.

# 1. Introduction

## 1.1. Imipramine mechanism

It is widely known that tricyclic antidepressants (TCA) are used to treat various psychological disorders; depression (Birkenhager, 2008), anxiety disorder (Stahl, 1998), eating disorders (Becker et al., 1999), and even chronic pain (McQuay *et al.*, 1996). Imipramine, a classic TCA, is prescribed widely for treatment. Therapeutic effects are seen after 2-4 weeks of chronic treatment with imipramine, the antidepressant influencing monoamine transmission in the brain affecting serotonergic, dopaminergic, and noradrenergic reuptake. Various aspects of the effects of TCAs and the wide spectrum of side effects are well documented but the mechanism of action is not fully understood. There are non-dopaminergic effects in non-target tissues including changes in neurotransmission, ion exchanges and fluxes, cytokine release, and influences on fluid membranes. This investigation looks at the effects of imipramine on haemolysis and attempt to establish a relationship between the observed findings and the mechanism of drug action.

Liquid membranes form as a result of ampiphillic molecules. Imipramine is a tricyclic amine with the hydrophilic and hydrophobic regions of a surface active drug. Surface active drugs form a barrier at the site of action preventing transport of other lipid permeable substances and solutes (Nagappa *et al.*, 2003). The 'liquid membrane hypothesis of drug action (Srivastava *et al.*, 1981; Nagappa *et al.*, 2003), a recently proposed theory, provides a new principle to explain the mechanism of TCA.

The ampiphillic structure, in an aqueous medium, forms a liquid membrane at surface-membrane interface by a surfactant layer lowering the interfacial tension between the two liquids (Nagappa *et al*, 2003). So imipramine molecules form a liquid membrane over the phospholipid bilayer of cells by surface adsorption (Fisar *et al.*, 2004). Kesting's liquid membrane hypothesis (Kesting *et al.*, 1968) states that when the concentration of a surface active drug increases, the interface becomes covered with surfactant molecules forming the liquid membrane, when the membrane is complete it is said to be at 'critical micelle concentration' (CMC). Formation of liquid membranes may influence movement of permeants through the surfactant layer thus affecting the mechanism of surface active drugs like imipramine; Nagappa *et al* (2003) demonstrated imipramine reducing permeability of biogenic amines such as dopamine, 5-HT, and norepinephrine, it also reduced permeability to cations when the concentration of imipramine was higher than its CMC. Research of organic molecules in unstirred layer theory (Pohl *et al.*, 1998) showed that transport of permeants can be reduced by surface adsorption dependant on size of the membrane formed and the solute diffusion coefficient. This supports the hypothesis that their surface active properties affect the mechanism; the high concentration of drug allows for multiple layers of liquid membrane to form significantly reducing the movement of these permeants, if the monoamines are prevented from entering storage vesicles in the neuron their concentration increases in the synaptic cleft. Applying this to the imipramine *in vivo* mechanism demonstrates that liquid membrane formation at neurons reduces the re-uptake of monoamines enhancing the antidepressant effect. Similarly the liquid membrane theory can explain the imipramine side effect of postural hypotension. Liquid membrane formation around muscle cells decreases calcium cation movement into the muscles, this reduces the effect of calcium in muscles, that of excitation and contraction resulting in increased blood pressure; hence the surface activity of imipramine contributes to the lowering of blood pressure (Nagappa *et al*. 2003). The evidence of imipramine affecting cation movement indicates that it can significantly disrupt the regulatory volume control

mechanisms of cells as $Na^+$ and $K^+$ play an important role in the electrophysical properties of the cell membrane (Nagappa *et al*. 2003). If the drug was at CMC or higher the regulatory volume control mechanisms may be compromised.
Nagappa *et al. (2003)* studied the inhibitory effect on permeant movement by the formation of liquid membranes, using imipramine at a higher concentration than CMC; 2 x $10^{-4}$ M. It would be interesting to investigate the effects of partial liquid membrane formation when the drug is lower than its CMC, as it may affect permeants at less significant level, or have no effect at all. TCAs also show an effect on cell signalling and cell volume control (Nagappa, 2003; Yasuhara *et al.*, 1985). Imipramine mechanism influences ion exchangers at the membrane and impinge on cell regulatory volume control. It is important to understand the mechanisms involved in cell volume control as the action of imipramine affects this directly and so can influence the lysis of erythrocytes.

### 1.2. Cell volume control

Drug-induced haemolysis of RBCs can arise from immune and non-immune responses. This study focuses on non-immune related imipramine-induced haemolysis. This non-immune haemolysis may occur by disruption of CVC mechanisms. The membrane of erythrocytes is an asymmetrically arranged phospholipid bilayer with unesterified cholesterol dispersed between the phospholipid molecules. The level of cholesterol amongst the phospholipids affects the fluidity of the membrane. Rigidity is influenced by the presence of membrane proteins within the lipid bilayer, there are three functional sets; catalytic, structural and receptor (Ballas & Krasnow, 1980). Fundamental in CVC are structural proteins, spectrin and actin, providing the membrane with its viscoelastic properties (Ballas & Krasnow, 1980), allowing the cell to come under moderate stress and stretching to accommodate. Osmoregulation is a key process in all animal cells (Okada *et al.*, 2001); to survive, cells must avoid excessive alterations in cell volume in order to prevent damage to the membranes and integral structure of the cell (Lang *et al.*, 1998). Animal cells lack cell walls (Campbell & Reece, 2002) so if there is a continuous net uptake of water the cells swell and burst. Water permeation occurs via diffusion through the lipid bilayer and via aquaporins that are strictly for water permeation and not ions. The human cell contains many organelles and internal structures, even erythrocytes with a simple internal structure and few organelles contain a multitude of internal ions, transporter proteins, and many other membrane proteins (Ballas & Krasnow, 1980). The erythrocyte cell membrane is semipermeable so all the internal substance of the cell can create osmotic pressure to draw water into the cell in a hypoosmotic environment. The cell volume is controlled by changes in environmental ion concentration. The response to changes in the surrounding medium must be controlled as the membrane has no resistance to bursting once the environment has a lower osmotic pressure than the physiological level. Even if the extracellular environment remains stable the volume constancy must still be monitored by the cell as osmotically active molecules such as ions and other metabolites are constantly transported and changed by metabolic action (Lang *et al.*, 1998). Therefore actions of regulatory cell volume mechanisms are sustained constantly; the mechanisms involve cell membrane ion transport, management of the accumulation level of osmolytes and metabolites and the disposal of these in excess (Lang *et al.*, 1998).
The osmotic pressures of the cell are affected by $Na^+$, $K^+$, and $Ca^{2+}$ ions having different concentrations inside and outside the cell. This results in a membrane potential due to electropotential difference across the membrane. The $Na^+/K^+/Cl_2^-$ pump-leak model demonstrates how the ions are both actively transported and move passively in CVC.

This maintains the intercellular and extracellular electrically neutral charges; so if the external environment has a higher osmolarity than the internal environment the pump adjusts for this, the osmolarity of human blood is approximately 300mosm/L (Campbell & Reece, 2002). If anything disturbs the pump or inhibits its action the cell can no longer control the membrane potential and the cell lyses. McCarty & O'Niel (1992) states that $Ca^{2+}$ may play a central role in regulatory volume decrease mechanisms in most cells controlling hypotonic cell volume regulation. Although further research is needed to determine the exact dependency of cell CVC on calcium ions, their initial studies indicated a possible direct control of $K^+$: $Cl^-$ channels by calcium ions modulating K-Cl movement and so regulatory volume decrease. Although $Ca^{2+}$ does appear to have an effect on ion channels a phosphorylation step may be involved (Kracke & Dunham, 1990) and further biochemical action; CVC mechanisms are highly complex and are unlikely to be controlled solely by inner plasma membrane events, the signalling cascades are likely to involve biochemical pathways throughout the cell (McCarty & O'Niel, 1992). Studies have countered these results and shown the lack of calcium ion dependency of CVC and the importance of cyclicAMP in the regulation of this system (Borgese *et al.*, 1987). However calcium ions do play a role in the regulation of erythrocyte cell volume; The $Ca^{2+}$/$Mg^{2+}$/ATPase enzyme is responsible for transport of calcium ions across biological membranes (Heguilen *et al.*, 2008). The $Ca^{2+}$ pump in red blood cells (Fig. 1) acts as a $Ca^{2+}H^+$ antiporter (Niggli *et al.*, 1982) an electrogenic pump exchanging one calcium ion for a proton (Romero & Ortiz, 1988) so affecting the electrochemical arrangement at the membrane. Similarly cellular $Ca^{2+}$ and $Mg^{2+}$ levels in erythrocytes influence the $Na^+$/$K^+$ pump fluxes so can have a substantial effect on cell volume regulation (Fujise & Lauf, 1988). Two regulatory processes result in a dynamic equilibrium, regulatory volume increase (RVI) response via cyclicAMP regulated $Na^+$ influx and regulatory volume decrease (RVD) response by passive $Cl_2^-$ dependent $K^+$ efflux (Borgese *et al.*, 1987).

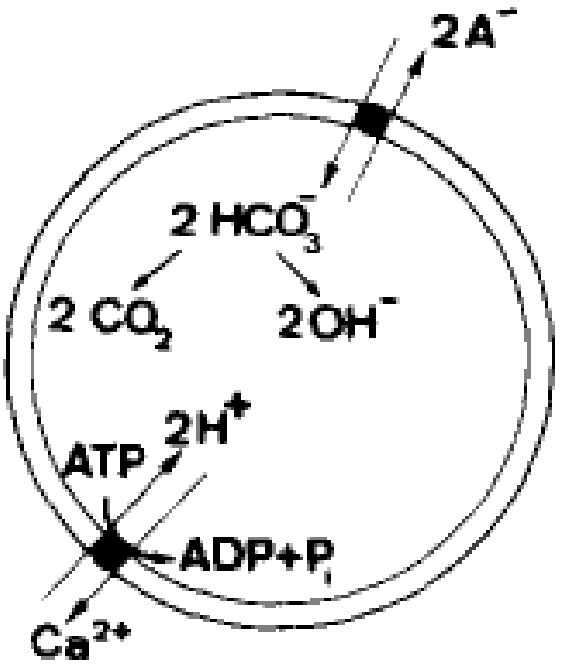


**Fig. 1. $Ca^{2+}H^+$ antiporter.** Schematic representation of $Ca^{2+}H^+$ antiporter anion fluxes in red blood cell. (Adapted: Niggli *et al.*, 1982)

RVI and RVD mechanisms are thought to be signalled by intracellular events triggering the membrane proteins and transport systems and resulting in second messenger cascades. The mechanisms result in adjusted intracellular ion composition and consequently altered cell membrane potential. These regulatory systems are involved in osmoregulation, but can also affect epithelial transport and hormone release, and impaired RVI and RVD can play a role in pathophysiological apoptosis and necrotic cell death (Lang *et al.*, 1998).

### 1.3. Aims and objectives

Research has reported that imipramine increases haemolysis of erythrocytes (Ahyayauch *et al.* 2004; Dutta e*t al.* 2007) the aim of this study was to support or refute these findings based on new data. The objectives of the experiment were 1) elaborate on the possible mechanisms of CVC, 2) investigate the influence of imipramine on these mechanisms, 3) determine if imipramine has a concentration-dependant haemolytic effect on RBCs, and 4) investigate the effect of prolonged exposure to the drug. The null hypothesis is that imipramine HCL has no effect on erythrocyte haemolysis. The alternative hypothesis is that imipramine HCL will have an effect on erythrocyte haemolysis.

## 2. Materials and methods

### 2.1. Stock solutions

| PBS Dilution | Proportion of PBS Stock (ml) | Proportion of Distilled water (ml) | NaCl concentration (mM) |
|---|---|---|---|
| 0 % | 0 | 100 | 0 |
| 10 % | 10 | 90 | 14 |
| 20 % | 20 | 80 | 28 |
| 30 % | 30 | 70 | 42 |
| 40 % | 40 | 60 | 56 |
| 50 % | 50 | 50 | 70 |
| 60 % | 60 | 40 | 84 |
| 70 % | 70 | 30 | 98 |
| 80 % | 80 | 20 | 112 |
| 90 % | 90 | 10 | 126 |
| 100 % | 100 | 0 | 140 |

**Table 1. Series dilution proportions**

#### 2.1.1. Phosphate buffered saline

1 buffer tablet (PBS, Dulbecco 'A' Tablet, *Oxoid Ltd.* Basingstoke, Hampshire, UK) was dissolved in 100 ml distilled water using an agitator to ensure all the powder was dissolved. This produced 100 % PBS solution with a concentration of approximately 140 mM NaCl at pH 7.4. Using the 100 % PBS stock solution a series dilution was carried out (Table 1) to produce a NaCl concentration range of approximately 0 mM-140 mM.

### 2.1.2. Imipramine stock solution

Imipramine HCL ($C_{19}H_{24}N_2$ HCl MW 316.87) was obtained from *Sigma-Aldrich* (Poole, Dorset, UK) and a stock solution prepared. Blood is denser than water so any concentration of drug to be made up with water must be adjusted for this density difference. Calculations for the stock solution were carried out as follows, the imipramine HCL LD50 [Rat] was used as the starting concentration for the calculations: Imipramine HCL LD50 [Rat] = 305 mg/Kg. The average human is 80 Kg and has approximately 7 pints of blood. So of 80 Kg 7 % is blood; 80/100 x 7 = 5.6 Kg of blood. With a blood density of 1.06 g/cm$^3$ and a blood-water ratio of 1.06:1.00 the volume of blood can be calculated. 5.6 Kg x 1.06 = 5.936 L. So the LD50 of imipramine is 305 mg in 80 Kg = 24.4 g in 5.936 L of blood. The drug will be made up in 1.1 ml volume in total so 24.4 x 1.1/5936 = 4.52 mg/1.1 ml. The volume of drug used in each trial is 50 µl so 4.25 x 20 = 90.4 mg/ml. To make up a 10 ml stock solution at this LD50 concentration 90.4 x 10 = 904.0 mg/10 ml.

904.4 mg of imipramine HCL was measured out accurately using a closed weighing balance. In a volumetric flask the weighed imipramine was made up to 10 ml with distilled water and shaken until all the powder was dissolved. This provided the stock solution at 90.4 mg/ml imipramine HCL. Further dilutions were made up with a dilution factor of 10 to provide a range of weaker solutions down to 0.0904 mg/ml.

### 2.2. Cell preparation

Horse erythrocytes were obtained from *Oxoid Ltd* (Basingstoke, Hampshire, UK). The cells were supplied in citrate buffer from a live donor and were defibrinated to prevent clotting. By obtaining the sample from a living donor ensured cells were in good condition to give clear and valid results. The cells were washed and resuspended in physiological saline; the blood was kept on ice before and during procedure. Using 10 ml Fallon tubes the required amount of blood to be washed was placed into a *Denley B5400 Benchtop* Centrifuge. The blood sample was centrifuged at 2000 rpm for 5 min. The tubes were removed from the centrifuge without disturbing the supernatant. The supernatant was pipetted off and discarded. Phosphate Buffered Saline (PBS) was added to maintain the volume of blood in the tube. The PBS had been stored on ice to prevent heat shocking the cells. The sample was then resuspended by gently rolling the tube in the palm of the hand to prevent damaging the cells by shaking. This process was then repeated until the supernatant was clear and no more lysed cells were settling out of the solution. A fresh sample of the donated blood was washed for every laboratory session.

### 2.3. Haemolysis assay

In order to draw conclusions from both time trial and dosage experiments with the drug, a control curve was produced. The haemolysis assay was carried out with every new batch of blood and on consecutive laboratory sessions to obtain a standard curve for all experiments carried out in any one laboratory session. For the dosage trials control 1.05 ml of each of the serial dilutions of PBS were pipetted out into 11 correspondingly labelled Eppendorf tubes, giving a NaCl concentration range of approximately 140 mM – 0 mM. 50 µl of the washed blood sample was added to each tube and the tube sealed firmly, this step was carried out quickly to ensure the incubation time was even for each tube. The tubes were inverted gently and allowed to incubate for 3 min then centrifuged (*Microcentrifuge 5415D*) at 13,000 rpm for 5 min. Without disturbing the pellet the supernatant of each tube was pipetted into a 1 ml cuvette. Using a *Thermospectronic Heliose* spectrophotometer the absorbance of each supernatant was

read at 540 nm and recorded in a table. The spectrophotometer was blanked with physiological saline before reading the samples. The process was repeated twice more to obtain triplicate results for means calculation. Absorbance values were converted into percentage haemolysis using the mean of 100 % lysis controls. This was used to calculate haemolysis for all the experiments using the relevant control data for the specific experiment, % Haemolysis = (absorbance of sample/average absorbance of 100 % haemolysis control) x 100.
For the time trial control 1.05 ml of 100 % PBS was pipetted into 18 Eppendorf tubes and 50 µl of blood added. The tubes were inverted every 5 min to maintain the cells in suspension. At 10 min intervals for a 60 min period three tubes were centrifuged and the absorbance reading taken as with the dosage haemolysis assay. Converting absorbance into percentage haemolysis provided triplicate results of the amount of haemolysis at 10 minute intervals over an hour.

### 2.4. Experiments

#### 2.4.1. *Imipramine concentration trials*

The experiment was run as with the haemolysis assay above, but only 1 ml of each PBS solution was added to the Eppendorf tubes. 50 µl of the drug solution was added to each tube before adding the 50 µl of washed blood. The tubes were incubated for 3 minutes and centrifuged at 13,000 rpm for 5 min in a *Microcentrifuge 5415D.* A range of drug concentrations was tested, each dose experiment repeated three times to calculate averages. Corresponding triplicate haemolysis controls were carried out with 50 µl of PBS in place of the drug as stated in haemolysis assay method.

#### 2.4.2. *Incubation-time trials*

Only 100 % PBS solution was used in the time trials. 1 ml of 100 % PBS was pipetted into 18 Eppendorf tubes, 50 µl of the concentration of drug being tested and 50 µl of blood was pipetted into each tube. The incubation times increased over an hour with 10 min intervals, obtaining readings at 10, 20, 30, 40, 50, and 60 min. The tubes were centrifuged and the readings taken of the supernatants as with the haemolysis assay. Three tubes were tested for each incubation time to provide triplicate results. The tubes were all inverted gently every 5 min to keep the cells in suspension. The time trial was run for all concentrations of drug to see the effect of time on the haemolysis of the cells at different dosages. Corresponding triplicate haemolysis controls were carried out with 50 µl of PBS in place of the drug as stated in haemolysis assay method.

### 2.5. Statistics

% Haemolysis was calculated for all data and expressed as a mean of the triplicate readings. Data of imipramine concentration dependent haemolysis was analysed using a paired t-test to compare treatments and a one-way ANOVA to compare specific data points, time dependent haemolysis results were analysed using one-way ANOVA. Time and dose effect were compared using a two-way ANOVA. The program used was SPSS 16.0. Differences were significant if $p < 0.05$.

## 3. Results

The haemolysis assay was as expected in trials with untreated cells; 100 % Haemolysis around 70-84 mM NaCl and below. Variation was due to the variation in the blood samples; erythrocyte count, age of the blood in situ, and the washing procedure. This

batch effect can influence all the results so a corresponding haemolysis assay control is given with each new batch of results.

### 3.1. Imipramine concentration dependent haemolysis

The dosage trials of imipramine HCL varied in effects; 90.04 mg/ml gave 100 % lysis at all NaCl concentrations with significantly greater lysis than the control in all readings (t-test, P <0.05) (Fig. 3). 9.04 mg/ml and 0.904 mg/ml both caused greater lysis than the control, but were close to the results for the control. 0.0904 mg/ml showed unusual results; this lowest concentration appeared to cause less haemolysis than the control at lower [NaCl] when the cells were under more stress. The control caused 100 % haemolysis at 14 mM NaCl whereas the 0.0904 mg/ml drug trial caused 96 % haemolysis (Fig. 2). This result was from a triplicate set of results and when the repeats were split the same lysis effect was seen with the drug causing 93 %, 98 %, and 96 % haemolysis respectively at 14 mM NaCl, all significantly different from the control (t-test, P = 0.003).

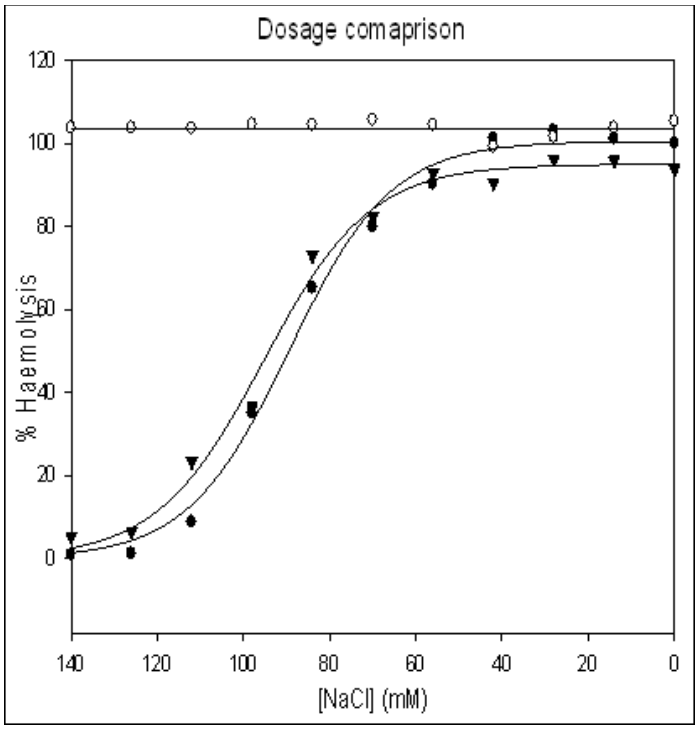


**Fig. 2. Effect of lowest and highest imipramine concentration on haemolysis of RBCs in varied extracellular osmolarity**. Control drug-free dilution series (●), 90.4 mg/ml (○), 0.0904 mg/ml (▼). Cells were incubated for 3 min. Data plots are means as a percentage of 100 % lysis control, n = 3. 0.0904 mg/ml caused significantly less lysis compared to control at lower [NaCl] where the cells were under greater stress, 14 mM (t-test P <0.05.

This result diverges from the pattern of the other drug concentrations which all caused relatively greater lysis than the control, so a repeat series of triplicate tests were carried out (Fig. 3). In repeat one the control caused 98 % haemolysis at 14 mM NaCl whereas the 0.0904 mg/ml drug trial caused 95 % haemolysis, significantly less lysis seen with the drug trial (t-test, P = 0.012) (Fig. 3A), when the triplicates were split again the same lysis effect was seen with the drug causing 94 %, 96 %, and 96 % haemolysis respectively at 14 mM NaCl. A further repeat also showed 0.0904 mg/ml having a lower haemolytic effect; at 0 mM NaCl, the control gave 100 % lysis but the drug only caused

82 % lysis (Fig. 3B). The two repeats are not comparable as they were carried out during different lab sessions with different blood samples, the relationship in the result is clear. In both repeats the drug caused less haemolysis than the respective control at low NaCl concentration when the cells were under greatest stress.

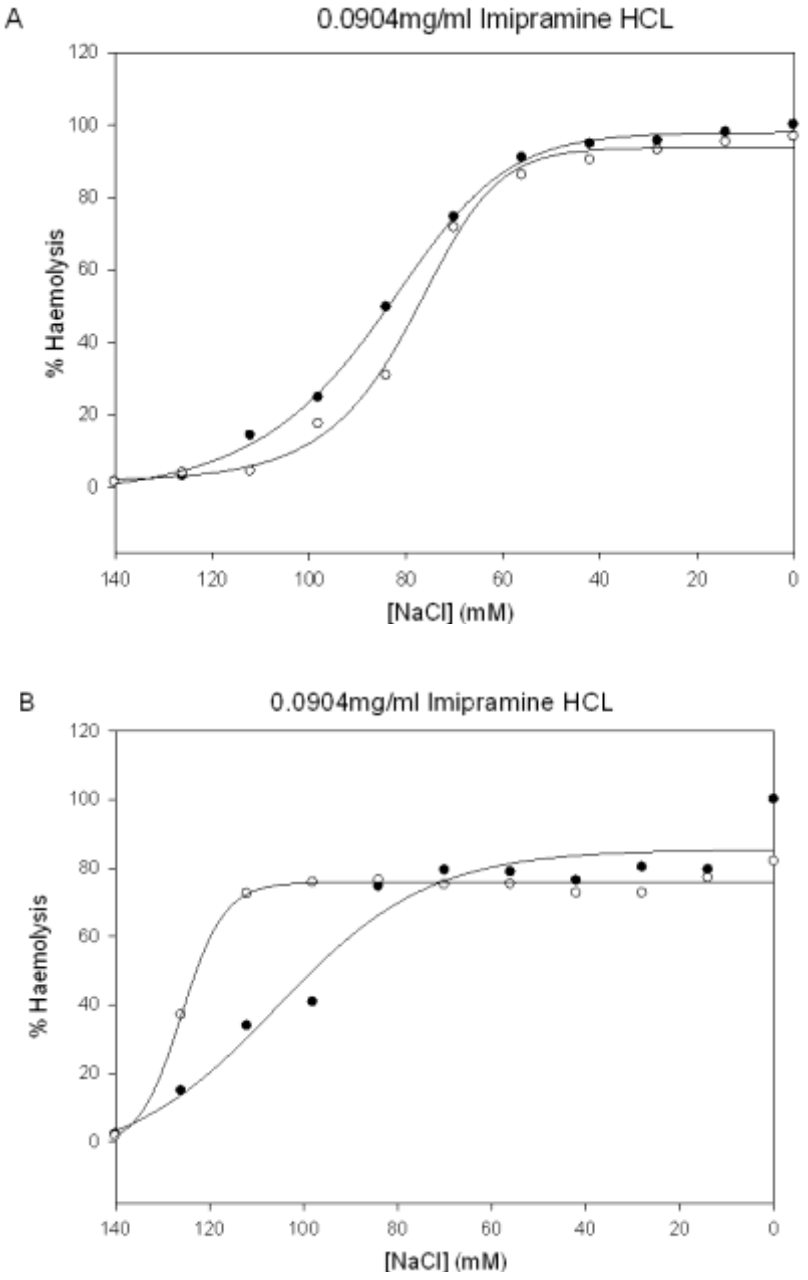


**Fig. 3. Stabilising effect of 0.0904 mg/ml imipramine on RBC haemolysis in varied extracellular osmolarity.** Drug-free control (●), 0.0904 mg/ml (○). Cells were incubated for 3 min. Data plots are means as a percentage of 100 % lysis control, n = 3. 3A 0.0904 mg/ml vs. control P = 0.012 (t-test).

Evidence of imipramine dosage affecting erythrocyte haemolysis is demonstrated by the fact that at 100 % PBS with 5 min incubation time the higher dosages of imipramine resulted in higher percentage lysis (Fig. 4). The graph shows the concentration-dependent effect of the drug up to 45.20 mg/ml. At higher concentrations this effect

increases steeply, indicated by the slope of the line (Fig. 4). After 5 min incubation 100 % lysis is shown by 54.24 mg/ml and higher.

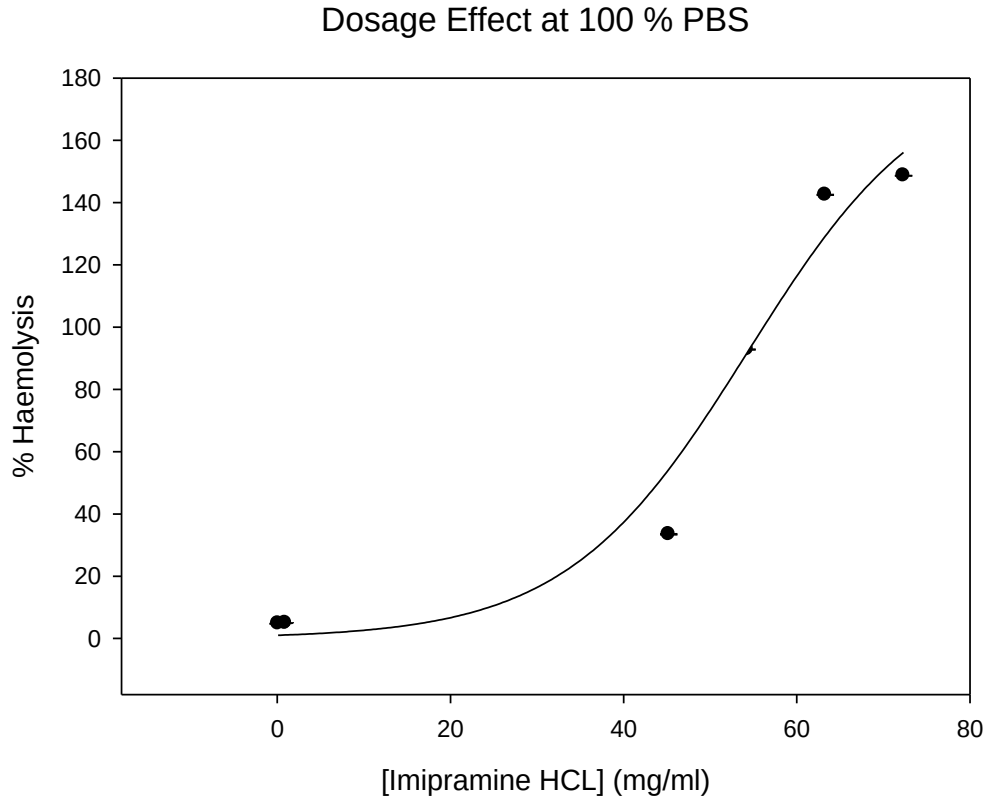


**Fig. 4. Effect of varied imipramine concentration on RBC haemolysis at 100 % PBS.** Rsq = 0.968 nonlinear regression. f= 184/(1+exp(-(x-x0)/10)). Cells were incubated for 5 min. Data plots are means as a percentage of 100 % lysis control and SEM, n = 3. P <0.05 (one-way ANOVA).

### 3.2. Incubation-time dependent haemolysis

The different concentrations of imipramine were investigated over a long incubation time to see the effect of different doses with extended incubation periods. The control haemolysis assay for the time trials indicated that lysis increases over time without any drug present. The control is used as a baseline haemolysis over time to compare with the haemolysis caused by increasing incubation time with drug. An incubation time trial was not carried out for 90.4 mg/ml as it gave 100 % lysis after just 3 min incubation shown in the dosage experiment (Fig. 2). The time trial carried out for 0.0904 mg/ml supported the results from the dosage experiment. This low concentration of drug caused lower haemolysis than the control across the 60 min incubation time. After 10 minutes incubation the control caused 3 % lysis, whereas the 0.0904 mg/ml drug trial only caused 1 % lysis (Fig. 5).

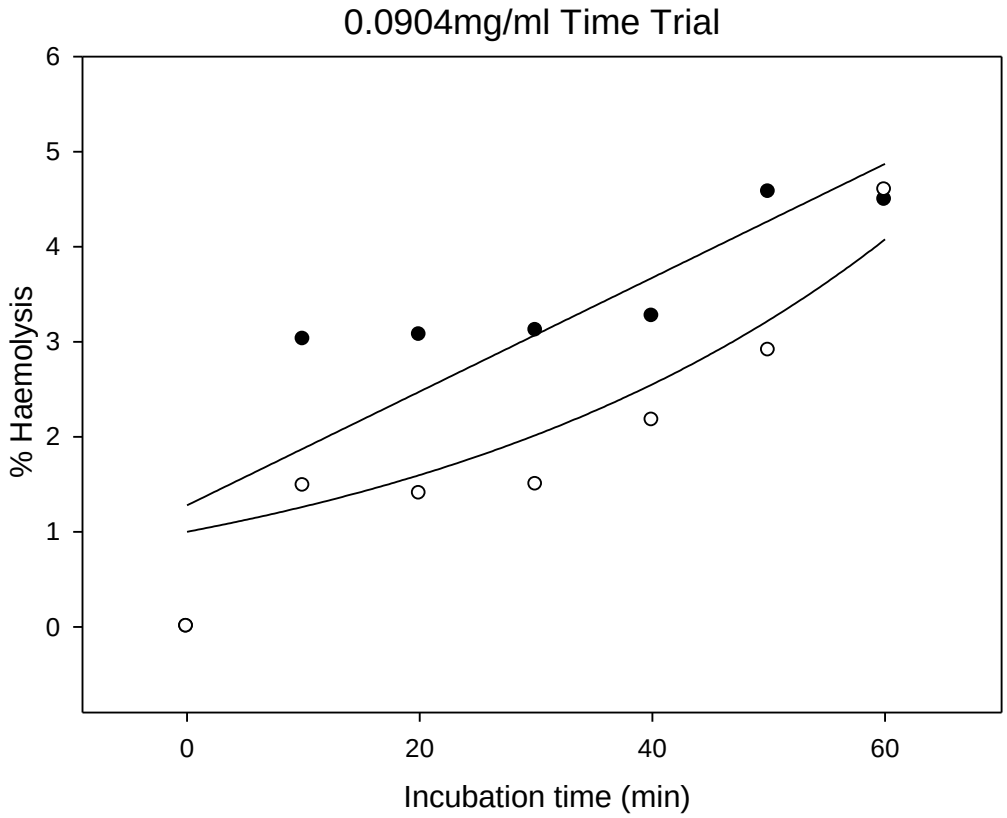


**Fig. 5. Effect of 0.0904 mg/ml imipramine over 60 min incubation at 100 % PBS.** Drug-free control (●), 0.0904 mg/ml (○). Cells were incubated for 5 min. Data plots are means as a percentage of 100 % lysis control, n = 3. 0.0904 mg/ml imipramine caused significantly less lysis vs. control (P <0.05 ANOVA).

The time trials at higher concentrations (Fig. 6) showed corresponding results to the control; the higher concentrations of imipramine HCL showed a regular pattern of results with the percentage haemolysis increasing with increasing incubation time. The higher concentrations of drug reached 100 % haemolysis after a shorter incubation time, causing more lysis over a shorter time than the lower concentrations. Above 45.20 mg/ml the results were predictable with 100 % haemolysis being reached within the first 10 min of incubation, so the results for the time trials of 54.24 mg/ml, 63.28 mg/ml, and 72.32 mg/ml imipramine looked much the same as 45.20 mg/ml (Fig. 6).
The 0.904 mg/ml and 9.04 mg/ml concentrations of imipramine both caused <7 % lysis over the entire incubation time with haemolysis values close to those of the control which caused <5 % lysis over the entire incubation time. These low imipramine concentrations of 0.904 mg/ml, 9.04 mg/ml and 18.08 mg/ml were not found to be significantly different to the control, the haemolysis for these concentrations were consistently similar to the haemolysis caused by the control over 60 min incubation. Both time and dosage effects were compared; dosage had a more significant effect on haemolysis that time (ANOVA, P = 0.008).

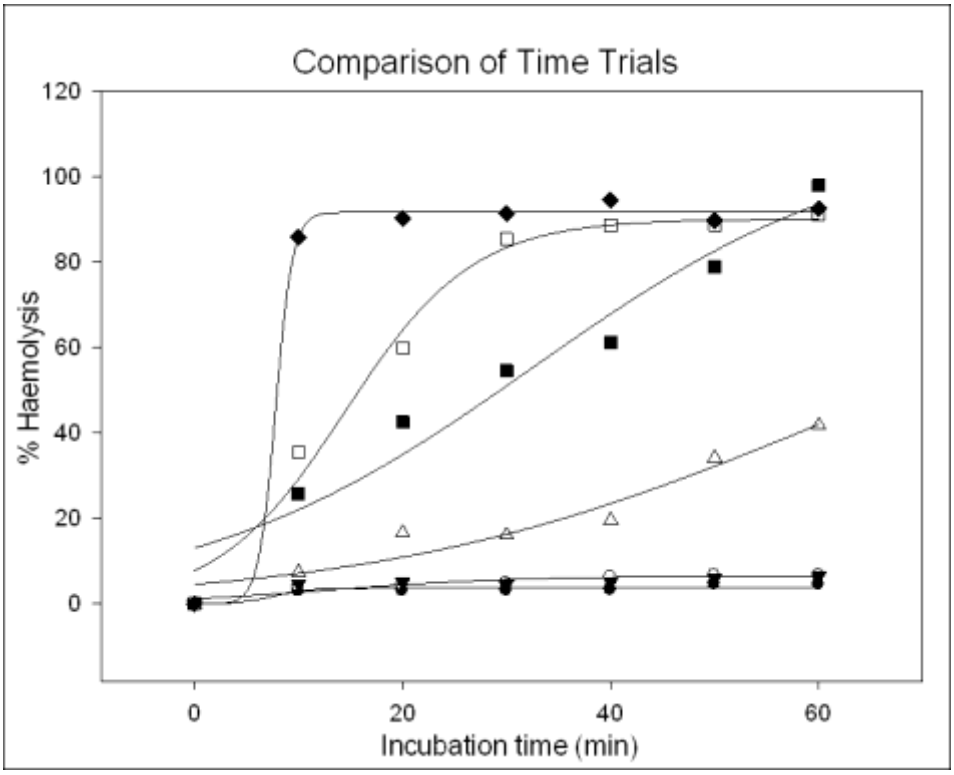


**Fig. 6. Effect of varied imipramine doses over 60 min incubation at 100 % PBS.** Drug-free control (●), 0.904 mg/ml (○), 9.04 mg/ml (▼), 18.08 mg/ml (Δ) 27.12 mg/ml (■), 36.16 mg/ml (□), 45.20 mg/ml (♦). Cells were incubated for 5 min. Data plots are means as a percentage of 100 % lysis control, n = 3. 27.12 mg/ml and higher were significantly different to the control (ANOVA, P = <0.05).

## 4. Discussion

This study clearly demonstrated that imipramine effects lysis of horse erythrocytes. Both dosage and exposure influenced the rate and level of haemolysis, indicating that imipramine induced haemolysis is both concentration and time dependent. Low concentrations of imipramine had a stabilising protective effect against haemolysis. All experiments were in triplicate, ensuring valid results unaffected by human error and batch variables. The haemolysis assay detailed in Watts & Handy (2007) supports the validity of the method used so valid conclusions may be drawn from the results.

### 4.1. Clinical implications

Clinical implications arise from the concentration and time-dependent haemolytic effects of imipramine. Hospitalised depressed patients often have doses of imipramine increased up to 300 mg/day over a long period (Birkenhager *et al.*, 2005). In an 80 Kg human this equates to 0.05 mg/ml circulating dose, which according to the results is insufficient to cause haemolytic effects at normal physiological blood osmolarity; the lowest dose investigated, 0.0904 mg/ml, in fact showed a stabilising effect against haemolysis. Even at the maximum prescribed dose a patient would have a 10 fold protective margin (0.5 mg/ml) before suffering any haemolytic effects, as 0.904 mg/ml caused <5 % haemolysis immediately after dosing at 100 % PBS (Fig. 4). However, if the patient was in trauma with abnormally dilute blood osmolarity, an overdose could cause serious instantaneous effects; 70 mM NaCl 100 % haemolysis was caused by 0.904 mg/ml imipramine. Cardiovascular illness has been associated with imipramine

(Jefferson, 1975) leading to disturbance in osmoregulatory systems, even a few percent of hypo-osmotic stress could significantly increase haemolysis levels at high imipramine dose; at 112 mM NaCl 0.904 mg/ml caused >10 % haemolysis. Progressive increase in imipramine-induced haemolysis over time (Fig. 6) indicates long term exposure to an overdose could have serious implications; 7 % haemolysis was caused after 60 min.

### 4.2. Imipramine concentration dependent haemolysis

The results show that high concentration of imipramine, 90.4 mg/ml caused complete haemolysis despite the osmotic environment. Even a 10 fold reduction in concentrations of imipramine caused high levels of cell lysis, compared to controls. This indicates the drug either has a toxic effect on cell membrane stability or disrupts integral cell volume control mechanisms. The interesting result of 0.0904 mg/ml imipramine causing less lysis than the control (Fig 3) may be explained by the surface action of the imipramine molecules. Formation of liquid membranes by ampiphillic molecules may explain the inhibiting effect of imipramine on monoamine reuptake at synapses. At concentrations significantly higher than CMC imipramine forms multiple surfactant layers at the interface of the synaptic terminal and the cleft, this extra membrane disrupts movements of permeants. Hence neurotransmitters, like noradrenaline, cannot be absorbed into transport vesicles and remain in the synaptic cleft increasing noradrenergic action; this ultimately propagates a therapeutic effect since depression is associated with decreased noradrenergic action at the synapses. Liquid membranes can develop at the interface of any two liquids, since erythrocyte membranes closely resemble that of nerve cells (Rybakowski, 1990) imipramine molecules may form liquid membranes around the erythrocytes in solution with those cells.

Since at and above CMC a complete liquid membrane is formed by the surfactant imipramine molecules, then below CMC only a partial liquid membrane may form, in keeping with the Kesting hypothesis. A low concentration of imipramine, 0.0904 mg/ml, appears to have a stabilising lytic effect on RBCs under stress, lending credence to the effect of liquid membrane formation. The CMC for imipramine is $1.48 \times 10^{-4}$ M so 0.0904 mg/ml or $2.8 \times 10^{-4}$ M is very close to it, indicating that the molecules form a thin liquid membrane but cannot build up layers of surfactant to disrupt movements of permeants through aquaporins, ion exchangers and the bilayer. The liquid membrane explains the stabilising effect of imipramine; the partial liquid membrane reinforces the erythrocyte membrane. Cells placed in a hypotonic solution are under stress, the liquid membrane reduces the hydraulic conductivity co-efficient, allowing for greater viscoelasticity and greater resistance to stress induced haemolysis.

Literature supports the stability theory; the 'liquid membrane hypothesis of drug action' developed as a result of many studies finding drugs interacting with membranes (Srivastava, 1981). Some studies found that surface active drugs like neuroleptic drugs interact with and can exert a stabilising effect on membranes (Seeman & Bialy, 1963; Seeman, 1966) including erythrocyte membranes (Seeman & Weinstein, 1966). A study by Srivistava *et al.* (1982) found specifically that imipramine *in vitro* formed a liquid membrane with a supporting membrane and reduced permeability to cations at concentrations above CMC. Further support for imipramine showing a stabilising effect on lysis of cells under stress comes from Yashura *et al.* (1980). This current study found that at $2.8 \times 10^{-4}$ M (0.0904mg/ml) imipramine protected against hypotonic haemolysis but above this it facilitated haemolysis. Yashura *et al.* (1980) found that imipramine protected rat erythrocytes from hypotonic haemolysis at concentrations from $1 \times 10^{-5}$ M to $4 \times 10^{-4}$ M but facilitated haemolysis at concentrations above $4 \times 10^{-4}$ M.

Similarly support from the literature is found in a study investigating the effect of neuroleptic drugs on cell membranes and lithium transport in erythrocytes (Azizabadi-Farahani *et al.*, 1996). Recently Nagappa *et al.* (2003) found that imipramine at concentrations of $2x10^{-4}$ M, above CMC, forms a liquid membrane in series with a supporting membrane significantly reducing the transport of adrenaline. As with this study, they also found a proportional decrease in hydraulic conductivity co-efficient as drug concentration increased towards CMC. Hence a drug concentration close to CMC should show less sensitivity when submitted to a hydraulic gradient than concentrations significantly higher than CMC, borne out in this study as 0.0904 mg/ml or $3.2x10^{-4}$ M is close to the imipramine CMC of $1.48x10^{-4}$ M (Srivastava *et al.*, 1982). The results show that higher concentrations of imipramine did not reduce sensitivity to water permeability; instead haemolysis was facilitated by the drug, also in keeping with Nagappa *et al.* (2003) who found that higher concentrations of imipramine significantly above CMC had a relatively consistent hydraulic conductivity co-efficient. The conclusions in this study and in Nagappa *et al.* (2003) match those of Srivistava (1982) all of which show that as imipramine concentrations build to CMC there is a decrease in hydraulic conductivity co-efficient, which supports Kesting's liquid membrane hypothesis. Imipramine molecules at concentrations much higher than $1.48x10^{-4}$ M form a multilayer liquid membrane over erythrocyte membranes, which facilitate lysis by reducing transport of permeants such as cations involved in RVD mechanisms. At concentrations around $1.48x10^{-4}$ M imipramine molecules progressively form a liquid membrane over the surface of the erythrocyte reducing the hydraulic conductivity co-efficient and so stabilising the cell against lysis.

Liquid membrane formation is not the only possible explanation for the concentration dependent haemolytic effect of imipramine. It has been established that the ampiphillic nature of the imipramine molecule interferes with pumps on the erythrocyte membrane, if $Ca^{2+}$ movement out of the cell is interfered with high intracellular $Ca^{2+}$ levels could compromise the membrane integrity and cause lysis. $Ca^{2+}$ pump plays a role in RVD mechanisms; if impramine interferes with the pump the cell cannot adjust to prevent lysis in a hypo-osmotic environment, therefore haemolysis levels would increase with imipramine concentration. In the same way the results may be explained by the fact that imipramine interferes with $Na^{+}K^{+}$ pumps on the erythrocyte membrane, as imipramine concentration increases the damaging effect on cell volume control is intensified and haemolysis results. The cumulative effect of imipramine on various membrane ion exchangers could explain the increase in imipramine-induced haemolysis over time.

An alternative explanation for the results is found in Ahyayauch *et al.* (2004). In these experiments pH was not carefully controlled, Ahyayauch *et al.* (2004) showed data supporting these results, with imipramine causing destabilising effects to erythrocyte membranes, but they also found that pH affected the interaction with the membrane due to changes in the charge of the ampiphile. Imipramine molecules were found to interact more strongly and have the greatest protective effect against hypotonic haemolysis at close to neutral pH (Ahyayauch *et al.*, 2004). Therefore the stabilising effects seen here at 0.0904 mg/ml may be a result of the neutral pH of the solution increasing adsorption to the membrane while higher concentrations caused a change in pH, reducing adsorption to the membrane. Further research could investigate changes in pH as a result of increasing imipramine HCL concentration.

### 4.3. Incubation-time dependent haemolysis

The results for the time trials may be explained by imipramine molecules interfering with ion pumps disrupting cell volume control mechanisms. Liquid membrane formation may be the explanation for this disruption at the cell surface. Around the CMC at 0.0904mg/ml the membrane is stabilised and a lower percentage haemolysis is caused than when there is no drug present to stabilise the membrane by forming a liquid membrane. The higher concentrations of drug indicate increasing percentage haemolysis over time as a result of longer exposure to the drug forming multilayered liquid membranes over the cells. The time trials were carried out at 100 % PBS, approximately physiological saline, so cell volume control mechanisms were not activated, but over the incubation time the interference of the imipramine molecules with the movement of permeants and cations compromises the intracellular mechanisms leading to disruption and necrotic cell death – haemolysis. Nagappa *et al.* (2003) support this theory, that high concentrations of imipramine disrupted the movement of cations and biogenic amines. The influence of the orientation of the ampiphillic molecules in liquid membranes and how this affects the permeability of ions was investigated in the Srivistava (1982) study. They found that both orientation of molecules and hydrophilicity of ions reduced permeability to cations.

## 5. Conclusion

The results and the relevant literature indicate with robust support that imipramine does have an effect on erythrocyte haemolysis. Liquid membranes are intrinsic to both imipramine mechanism of inhibiting movement of biogenic amines at high concentrations, and to the membrane stabilising effect at low concentrations. Overall it is important to note that the surface activity of imipramine influences its mechanism and can have implications for research involving ion exchange and flux at cell membranes. Conclusions regarding surface activity of imipramine on erythrocytes and support from other studies help understand imipramine mechanism and the effects the molecule has on nerve conduction. Temperature was not closely controlled in either time trials or dosage experiments, and was approximately room temperature; 20$^{o}$C, further research should investigate the effects of liquid membrane formation with the cells under temperature stress, and the effect of pH on imipramine-induced haemolysis.

Further research needs to look specifically at the effects of liquid membranes on both passive and active transport across cell membranes to investigate imipramine effect on nerve conduction. This study should be repeated looking at concentrations of imipramine HCL far below its CMC to investigate the effects on lysis as concentration rises to CMC. Further investigation may support the theory that progressive formation of liquid membranes by surface active drugs can exert a stabilising effect on membranes, and would verify findings from other studies on the liquid membrane hypothesis.

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